TABLE II. Positive Mucin Clot Test in Supernate of Cultures of Chick Embryo Brain Tissue.

	Tubes after 10 days		Tubes after 30 days		Tubes after 47 days	
Tubes	Good growth	Mc +	Good growth	Mc +	Good growth	Mc +
10	8	4	7	6	3	3
10	6	5	6	6	4	3
20	11	7	13	9	8	7
12	10	2	8	6	8	8
20	18	0	18	3	18	15

the existence of a ground substance in the central nervous system, and half a century later the presence of a ground substance in the central nervous system has been denied (2). On the other hand, increased spreading (6) in the cerebral cortex was found after treatment with hyaluronidase(3). Recently the presence of mucopolysaccharide between the elements constituting the central nervous system was assumed on the basis of histochemical reactions(4.7). The demonstration by Hess of a positive periodic acid-Schiff (PAS) reaction between the cells of the central nervous system suggested the existence of a ground substance between neuroglia fibrils, axon terminations and dendrites(9). Since hyaluronidase did not alter the red coloration of the PAS reaction, it was assumed that the ground substance consisted of a neutral mucopolysaccharide and not of hyaluronic acid(9). Again more recently, the existence of a substance in the cerebral cortex of the cat which is susceptible to the action of hyaluronidase has been reported(1). The present results show that hyaluronic acid is produced in tissue culture by cells growing from explants of embryonic brain. This constitutes further evidence for the existence in the central nervous system of a hyaluronic acid containing ground substance.

Summary. The supernatant fluid obtained from tissue cultures of avian brain, on addition of 0.2 ml of 1 N acetic acid to 1 ml sample, continued to give for over 2 months a mucin clot the formation of which could be prevented by testicular hyaluronidase. This indicates production of hyaluronic acid in tissue culture by cells, other than neurons, growing from explants of embryonic brain.

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Effect of 5-Nitro and 5-Chloro Benzimidazole Derivatives on Heme Synthesis *in vitro*.* (23628)

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We have reported that certain 5-methylbenzimidazole derivatives, particularly 2-ethyl-5-methylbenzimidazole, 2,5 - dimethylbenzimidazole and 5,6 - dimethylbenzimidazole prevented incorporation of N^{15} from N^{15}-glycine into heme by chicken erythrocytes during *in vitro* incubation(1,2). Since the same benzimidazole derivatives also were found to inhibit virus duplication(3), we proposed that they might be inhibiting an important metabolic reaction fundamental to more than one biosynthetic process(1,2). They ap-

pear to affect an early step in glycine utilization since they prevented porphyrin synthesis from glycine but not from *delta*-aminolevulinic acid(4). We also have reported that 2, 5-dimethylbenzimidazole and 2-ethyl-5-methylbenzimidazole prevented production of liver tumors in rats fed azo dyes(5,6). These results have prompted investigation of additional 5-substituted benzimidazoles. Using chicken erythrocytes incubated with N^{15}-glycine as a test system, we have compared the effects on heme synthesis of a series of benzimidazole derivatives having nitro or chloro groups instead of a methyl in the 5 position. Similar studies with 5-hydroxybenzimidazole are also included.

Methods. 5-Nitrobenzimidazole, 2-methyl-5-nitrobenzimidazole and 2-ethyl-5-nitrobenzimidazole‡ were prepared by refluxing 4-nitro-*o*-phenylenediamine with the appropriate acid, according to the general procedure of Wagner and Millett(7). 5-Chlorobenzimidazole, 2-methyl-5-chlorobenzimidazole and 2-ethyl-5-chlorobenzimidazole‡ were prepared similarly from 4-chloro-*o*-phenylenediamine. 5-Hydroxybenzimidazole was kindly supplied by Dr. Karl Folkers of Merck and Co. Incubation of chicken blood and N^{15}-glycine, with and without the test compounds, hemin isolation and N^{15} analyses were carried out as described previously(1,2). Three 20 ml samples of the same blood were incubated at the same time, 2 test samples with a control.

Results. Data are presented in Table I showing N^{15} content of hemin isolated from chicken erythrocytes incubated with N^{15}-glycine (control), compared with hemin from erythrocytes incubated with N^{15}-glycine in the presence of 10 mg of the compound indicated (test). Lower N^{15} content is considered indicative of decreased heme synthesis, since samples of the same blood incubated with N^{15}-glycine under identical conditions, or incubated with non-inhibitory supplementary compounds, yield hemin with the same N^{15} content(8). Exp. 1 and 2 indicate that 2-ethyl-5-nitrobenzimidazole was considerably more inhibitory than 5-nitrobenzimidazole or the 2-methyl-5-nitrobenzimidazole. Similarly, in the 5-chloro series (Exp. 3 and 4), 2-ethyl-5-chlorobenzimidazole was considerably more inhibitory than the 5-chlorobenzimidazole or the 2-methyl-5-chlorobenzimidazole. All were more effective inhibitors than benzimidazole itself at this level (Exp. 7 and 8). It also appears that in each instance the 5-chloro derivative was somewhat more effective than the corresponding nitro analogue. 2-Ethyl-5-chlorobenzimidazole, which had almost complete inhibition at this level, was as effective as 2-ethyl-5-methylbenzimidazole, whereas 2-ethyl-5-nitrobenzimidazole was not (Exp. 5 and 6). Benzimidazole itself had only slight activity compared with the 2-ethyl-5-chloro- or 2-ethyl-5-nitro- derivative at this level, even though on a molar basis its concentration was somewhat greater (Exp. 7 and 8). 5-Hydroxybenzimidazole (Exp. 9) was no more effective than benzimidazole itself, and appeared to be even less inhibitory than benzimidazole at the 20 mg level(1,2).

Discussion. 5-Chloro and 5-nitro benzimidazole derivatives, like the 5-methylbenzimidazoles studied previously(1,2) inhibit incorporation of N^{15} from N^{15}-glycine into heme, whereas benzimidazole itself has comparatively little effect. With these compounds also, the 2-ethyl derivatives were more inhibitory than the 2-methyl. Tamm, *et al.*(9) found that 5-chlorobenzimidazole and 2-methyl-5-chlorobenzimidazole inhibited influenza B virus multiplication, and Schneider (10) noted that 6-nitro-benzimidazole (like benzimidazole and 2-ethyl-5-methylbenzimidazole) inhibited tobacco mosaic virus multiplication in tobacco leaf disk culture. Thus 5-chloro- and 5 (or 6)-nitrobenzimidazole,

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‡ These apparently have not been described previously. 2-Ethyl-5-chlorobenzimidazole melted 170°-171°; analysed (%): C, 59.8; H, 4.89; N, 15.56 (calculated: C, 59.8; H, 4.98; N, 15.51). 2-Ethyl-5-nitrobenzimidazole softened at 169° and melted 179°-180°; analysed (%): C, 57.2; H, 4.81; N, 21.72 (calculated: C, 56.5; H, 4.71; N, 21.98); analyses by Drs. Weiler and Strauss, Microanalytical Laboratories, Oxford.

TABLE I. Effect of 5-Nitro-, 5-Chloro-, and 5-Hydroxybenzimidazole Derivatives on Incorporation of N^{15} from N^{15}-Glycine into Heme by Erythrocytes of Chicken Blood during Incubation *In Vitro*.

Exp.	N^{15} concentration in hemin* Control	Test	Supplement†
	(atom % excess)		
1	.087	.054	5-nitrobenzimidazole
		.026	2-ethyl-5-nitrobenzimidazole
2	.073	.049	2-methyl-5-nitrobenzimidazole
		.020	2-ethyl-5-nitrobenzimidazole
3	.073	.029	5-chlorobenzimidazole
		.008	2-ethyl-5-chlorobenzimidazole
4	.074	.040	2-methyl-5-chlorobenzimidazole
		.010	2-ethyl-5-chlorobenzimidazole
5	.074	.009	2-ethyl-5-methylbenzimidazole
		.009	2-ethyl-5-chlorobenzimidazole
6	.096	.018	2-ethyl-5-methylbenzimidazole
		.036	2-ethyl-5-nitrobenzimidazole
7	.086	.079	benzimidazole
		.015	2-ethyl-5-chlorobenzimidazole
8	.067	.065	benzimidazole
		.026	2-ethyl-5-nitrobenzimidazole
9	.074	.072	5-hydroxybenzimidazole (10 mg)
		.062	" (20 ")

* Values on the order of .002 to .006 atom % excess indicate complete inhibition of N^{15} incorporation into heme(1,2).

† 10 mg of the compound to be tested was added to a flask otherwise identical with the control, except in Exp. 9, where 20 mg was also used.

like the 5-methyl derivatives, are inhibitory in two widely different biological systems, hemoglobin synthesis by avian erythrocytes *in vitro* and virus duplication.

Although methyl, chloro or nitro groups in the 5 position gave compounds having inhibitory activity greater than that of unsubstituted benzimidazole, it is of particular interest to note that 5-hydroxy substitution did not. 5-Hydroxybenzimidazole had only slight, if any, inhibitory activity at the 10 mg level, and appeared to have even less than that of benzimidazole at the 20 mg level. 5-Hydroxybenzimidazole occurs naturally as part of the Vit. B_{12}-like molecule of Factor III, isolated by Friederich and Bernhauer from fermented sewage(11). The biological function and significance of Factor III is unknown. One possible explanation of the inhibitory activities of 5-substituted benzimidazoles might be through blocking a reaction dependent upon the functioning of an active component containing a similar but not identical moiety. The inhibitory effect of 2,5-dimethylbenzimidazole was not altered by addition of Vit. B_{12}(1). This substance contains 5,6-dimethylbenzimidazole, a benzimidazole which also inhibited heme synthesis.

The relative ineffectiveness of 5-hydroxybenzimidazole as an inhibitor of glycine incorporation into heme, and its occurrence in the molecule of a complex naturally-occurring substance of unknown function, suggests the possibility that additional work with inhibitory benzimidazoles and Factor III might indicate whether Factor III plays a role in glycine utilization. Although inhibitory benzimidazoles appear to block a step early in the utilization of glycine, since they block porphyrin synthesis from glycine but not from *delta*-aminolevulinic acid(4), the nature of the step involved, and the relationship, if any, of benzimidazoles to the process, remain to be determined.

Summary. 5-Nitro-, 2-methyl-5-nitro-, and 2-ethyl-5-nitrobenzimidazole; 5-chloro-, 2-methyl-5-chloro-, and 2-ethyl-5-chlorobenzimidazole were prepared and their effects on incorporation of N^{15} from N^{15}-glycine into heme by chicken erythrocytes incubated *in vitro* were studied. Similar to the 5-methylbenzimidazole series studied previously, the 2-ethyl-5-nitro- was considerably more inhibitory than the 5-nitro- or 2-methyl-5-nitrobenzimidazole. Likewise in the 5-chloro series, 2-ethyl-5-chloro- was considerably more inhibitory than the 5-chloro- or 2-methyl-5-chlorobenzimidazole. 5-Chloro derivatives appeared to be more inhibitory than the corresponding 5-nitro analogues; 2-ethyl-5-chlorobenzimidazole was as effective as 2-ethyl-5-methylbenzimidazole, whereas 2-ethyl-5-nitrobenzimidazole was not. All were more effective than benzimidazole itself. Although methyl, chloro or nitro groups in the 5 position gave compounds having inhibitory activity greater than that of unsubstituted benzimidazole, 5-hydroxybenzimidazole was comparatively ineffective.

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